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Relaxant effect of *Crocus sativus* (saffron) on guinea-pig tracheal chains and its possible mechanisms

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Abstract

As indicated in ancient Iranian medical books, *Crocus sativus* has therapeutic effects on respiratory diseases. The relaxant effect of this plant has been observed also on smooth muscles in previous studies. Therefore, in this study the relaxant effects of aqueous-ethanolic extracts of *C. sativus* and one of its main constituents, safranal, were examined on guinea-pig tracheal chains. The relaxant effects of four cumulative concentrations of aqueous-ethanolic extract (0.15, 0.3, 0.45, and 0.60 g %) and safranal (0.15, 0.30, 0.45, and 0.60 mL 0.2 mg mL^{-1} solution) in comparison with saline, as negative control, and four cumulative concentrations of theophylline (0.15, 0.30, 0.45, and 0.60 mM), as positive control, were examined using guinea-pig precontracted tracheal chains. The tracheal chains had been precontracted by three different methods. Group 1 had been precontracted using 10 μM methacholine. The other two groups had been precontracted using 60 mM KCl at two different conditions: non-incubated tissues (group 2) and tissues incubated with 1 μM propranolol,1 μM chlorpheniramine and 1 μM atropine (group 3) (for each group, n = 6). In group 1 all concentrations of theophylline, extract and safranal showed significant relaxant effects compared with saline ($P < 0.05$ to $P < 0.001$). In group 2 theophylline, extract and safranal showed concentration-dependent relaxant effects also compared with saline ($P < 0.05$ to $P < 0.001$ for different concentrations except two low concentrations of safranal). However, in group 3 the extracts of *C. sativus* showed a weak relaxant effect ($P < 0.05$ only for the highest concentration). The effects of the last concentration of safranal (0.60 mL 0.2 mg mL^{-1} solution) in group 1, and all its concentrations in group 2 were significantly lower than those of theophylline ($P < 0.05$ to $P < 0.001$). In addition, the effects of safranal 0.45 and 0.60 mL 0.2 mg mL^{-1} solution in groups 1 and 2 were significantly lower than that of *C. sativus* extract. There were significant correlations between the relaxant effects and concentrations for extract, safranal and theophylline in all experimental groups ($P < 0.001$ for all cases). These results showed a potent relaxant effect of *C. sativus* on tracheal chains of guinea-pigs that was comparable to or even higher than that of theophylline at the concentrations used. The results indicated that safranal was, at least in part, responsible for the relaxant effect of *C. sativus*.

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Introduction

Crocus sativus L, commonly known as saffron, is a small perennial plant from the iridaceae family. It is cultivated in many places, but particularly in France, Spain, Sicily, and Iran. It has green, hairy leaves approximately one to one-and-a-half feet long and a funnel-shaped, reddish-purple flower. *C. sativus* is used in folk medicine as an antispasmodic, eupeptic, gingival sedative, anticatarrhal, nerve sedative, carminative, diaphoretic, expectorant, stimulant, stomachic, aphrodisiac and emmenagogue (Rios et al 1996). The stigma, also called the style (central part of the flower, the female sexual organ), is the part of the plant used medicinally. The main active constituents of this plant are picrocrocin and its derivatives including safranal, flavonoid derivatives and crocin (Tarantilis et al 1995).

Previous studies have shown different pharmacological effects for this plant including anticonvulsant (Hosseinzadeh & Khosravan 2002), antidepressant (Hosseinzadeh et al 2004), anti-inflammatory (Hosseinzadeh & Younesi 2002), antioxidant effect (Abe et al 1999), antitumour and radical scavenger effects (Abdullaev & Ferenkel 1992; Abdullaev 1993; Escribano et al 1996; Rios et al 1996), and learning and memory improving properties (Zhang et al 1994). Saffron extract also has chemopreventive and genoprotective effects and protects from genotoxin-induced oxidative stress in mice (Nair et al 1995; Premkumar et al 2001, 2003;

Abdullaev et al 2002). A blood pressure lowering effect (Rios et al 1996) and relaxant effect on vascular smooth muscle (Fatehi et al 2003) have been described also for this plant.

Therefore, in this study, we have investigated the relaxant effect of aqueous-ethanolic extracts from *C. sativus* and its main constituent, safranal, on guinea-pig tracheal chains, and its possible mechanisms have been examined.

Materials and Methods

Plant and extracts

C. sativus was collected from Torbat Heydarieh (east of Iran) and identified by Mrs Molaei at the Herbarium of the School of Agriculture, Mashhad University of Ferdowsi, where a voucher specimen was preserved (Herbarium No: 143-0319-1). To prepare the aqueous-ethanolic extract, 10 g chopped, dried plant was extracted with 25 mL distilled water and 25 mL ethanol by soxhlet apparatus. The solvent was then removed under reduced pressure and distilled water was added so that the plant ingredient concentration in the final extract was 10 g %.

Measurement of safranal and crocin

The amount of safranal and crocin in the extract was assessed using HPLC (Schimatzue, SSPD-10AVP with ODS column). A UV-vis detector set at a wavelength of 308 nm was used for assessing safranal, whilst for crocin the wavelength was set at 443 nm. The solvent was acetonitrile:distilled water (24:76, v/v) and the flow rate was 0.5 mL min^{-1}. The injected volume was 25 μL for the measurement of safranal and crocin. Using the standard curve of safranal and crocin (Fluka chemical AG, Switzerland), the presence of safranal and crocin was measured.

Tissue preparations

Male Dunkin-Hartley guinea-pigs (400–700 g) were killed by a blow on the neck and tracheas were removed. Each trachea was cut into 10 rings (each containing 2–3 cartilaginous rings). All the rings were cut open opposite the tracheal muscle, and sutured together to form a tracheal chain (Holroyde 1986). Tissue was suspended in a 10-mL organ bath (organ bath 61300, Bioscience Palmer-Washington, Sheerness, Kent, UK) containing Krebs–Henseleit solution of the following composition (mM): NaCl 120, $NaHCO_3$ 25, $MgSO_4$ 0.5, KH_2PO_4 1.2, KCl 4.72, $CaCl_2$ 2.5 and dextrose 11.

The Krebs solution was maintained at 37°C and gassed with 95% O_2 and 5% CO_2. Tissue was suspended under an isotonic tension of 1 g and allowed to equilibrate for at least 1 h while it was washed with Krebs solution every 15 min.

Protocols

The relaxant effects of four cumulative concentrations of aqueous-ethanolic extract (0.15, 0.30, 0.45, and 0.60 g %), safranal (Fluka chemical AG, Switzerland) (0.15, 0.30, 0.45, and 0.60 mL 0.2 mg mL^{-1} solution), four cumulative concentrations of theophylline anhydrous (Sigma Chemical Ltd UK) (0.15, 0.30, 0.45, and 0.60 mM) as positive control, and saline as negative control (0.6 mL) were examined. To produce different concentrations of aqueous-ethanolic extract, 0.15 mL 100 mg mL^{-1} concentrated extracts were added to a 10-mL organ bath respectively four times. For safranal 0.15 mL 0.2 mg mL^{-1} solution and for theophylline 0.15 mL 10 mM solution was added to the organ bath four times. The consecutive volumes were added to the organ bath at 5-min intervals.

The assessment of the relaxant effect of different solutions in each experiment were carried out as follows. The tracheal chains were first contracted with 10 μM methacholine. After achievement of maximum contraction, which was obtained after 7 min, four cumulative volumes from extract, safranal, theophylline or one volume of saline was added to the organ bath at 5-min intervals. The effect of different volumes of extract, safranal, theophylline or saline on contracted tissues was measured after exposing tissue to the solution for 5 min. The percentage effect of each volume of different solutions (decrease or increase of tracheal tone) was calculated in proportion to the maximum contraction effect induced by methacholine. A decrease in tone was considered as a relaxant (bronchodilatory) effect and expressed as positive percentage change in proportion to the maximum contraction; and an increase in tone was considered as a contractile (bronchoconstrictory) effect, which was expressed as negative percentage change (Martin et al 1994).

The relaxant effect of different solutions was tested with three different experimental designs (n = 6 for each group) as follows. Group 1: on tracheal chains contracted by 10 μM methacholine hydrochloride (Sigma Chemical Ltd, UK). Group 2: on non-incubated tracheal chains contracted by 60 mM KCl. Group 3: on tracheal chains incubated with 1 μM propranolol hydrochloride (Sigma Chemical Ltd, UK), 1 μM chlorpheniramine maleate (Sigma Chemical Ltd, UK) and 1 μM atropine sulfate (Sigma Chemical Ltd, UK) 30 min before beginning and during the testing relaxation of different solutions; in this series of experiments, tracheal chains were also contracted by 60 mM KCl.

The relaxant effect of theophylline and safranal was examined on groups 1 and 2 only. The relaxant effects in the three groups of experiments were examined in three different series of tracheal chains. All of the experiments were performed randomly with a 1 h resting period of tracheal chains between each two experiments, while washing the tissues every 15 min with Krebs solution. In all experiments responses were recorded on a kymograph (ET8 G-Boulitt, Paris) and were measured after fixation. The study was approved by the ethical committee of Mashhad University of Medical Sciences.

Statistical analysis

Data were expressed as mean ± s.e.m. Data of relaxant effects of different concentrations of extract and safranal were compared with the results of negative and positive control using analysis of variance. The data of relaxant effect between three

groups were compared using analysis of variance. The relaxant effects of extract, safranal and theophylline were related to the concentrations using least square regression. Significance was accepted at $P < 0.05$.

Results

Measurement of safranal and crocin

The amounts of safranal and crocin in the extract of *C. sativus* were 0.026 and 41.3%, respectively.

Relaxant (bronchodilatory) effect

In group 1, all concentrations of theophylline, extract and safranal showed significant relaxant effects compared with those of saline ($P < 0.05$ to $P < 0.001$). The effect of the highest concentration of safranal was significantly lower than those of theophylline ($P < 0.05$). In addition the effects of the two higher concentrations of safranal were significantly lower than that of extract in this group ($P < 0.05$ to $P < 0.01$) (Table 1).

In group 2, extract from *C. sativus*, safranal and theophylline showed relatively potent and concentration-dependent relaxant effects on tracheal chains of guinea-pig. The relaxant effects of all concentrations of extract, safranal and theophylline were significantly higher than those of saline ($P < 0.05$ to $P < 0.001$). Only the two low concentrations of safranal did not show significant relaxant effects (Table 2). However, the effects of all concentrations of safranal in group 2 were significantly lower compared with theophylline ($P < 0.05$ to $P < 0.001$) (Table 2). In addition, the effect of the two higher concentrations of safranal were significantly lower than those of extract ($P < 0.01$ for both cases).

In group 3, the extract of *C. sativus* showed relatively weak relaxant effect, and only the last extract concentration (0.60 g %) was significantly different compared with the effect of saline (Table 3).

Comparison of the relaxant effect between the three experimental groups

The relaxant effects of different concentrations of *C. sativus* extract and theophylline were not statistically different between groups 1 and 2. The relaxant effects of most concentrations of extract in groups 1 and 2 were significantly higher than those of group 3 ($P < 0.05$ to $P < 0.001$) (Figure 1). In addition, the relaxant effects of most concentrations of safranal in group 2 were statistically lower than those of group 1, except for the highest concentrations ($P < 0.05$ to $P < 0.01$) (Figure 1).

Correlation between concentrations of solutions and their relaxant effects

There were significant positive correlations between concentrations of the solutions with the relaxant effects of aqueous-ethanolic extract (0.927, 0.832 and 0.711 for groups 1, 2 and 3, respectively), safranal (0.728 and 0.782 for groups 1 and 2)

Table 1 Relaxant effect of aqueous-ethanolic extract from *Crocus sativus* and safranal in comparison with negative control (saline) and positive control (theophylline) in group 1 experiments (contracted tracheal chains with 10 μM methacholine, n = 6)

Different concn	Saline	Aqueous-ethanolic extract	Safranal	Theophylline
0.15	–	14.51 ± 1.51*	15.47 ± 4.51*	26.59 ± 6.35**
0.30	–	52.90 ± 6.16***	37.49 ± 8.43***	57.56 ± 6.85***
0.45	–	82.70 ± 5.00***†	48.63 ± 9.50***	73.59 ± 7.09***
0.60	1.45 ± 0.51	96.97 ± 4.23***‡‡	58.11 ± 9.50***†	87.40 ± 6.22***

Values are presented as mean ± s.e.m. of percentage change in proportion to the maximum contraction induced by 10 μM methacholine. The unit of concentration for extract was g %, for safranal volume (mL) of 0.2 mg mL^{-1} solution, and for theophylline mM. For saline only one volume (0.6 mL) was used. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with saline; †$P < 0.05$, compared with theophylline; ‡$P < 0.05$, $P < 0.01$ compared with safranal.

Table 2 Relaxant effect of aqueous-ethanolic extract from *Crocus sativus* and safranal in comparison with negative control (saline) and positive control (theophylline) in group 2 experiments (non-incubated preparation contracted by 60 mM KCl, n = 6)

Different concn	Saline	Aqueous-ethanolic extract	Safranal	Theophylline
0.15	–	14.11 ± 5.89*	4.45 ± 1.70†	18.91 ± 1.86*
0.30	–	44.95 ± 9.78***	11.01 ± 2.51††	52.36 ± 2.20***
0.45	–	67.72 ± 10.75***‡‡	25.64 ± 4.74*††	70.13 ± 0.78***
0.60	1.02 ± 0.52	86. 61 ± 9.80***‡‡	37.54 ± 8.64*††	83.70 ± 1.09***

Values are presented as mean ± s.e.m. The unit of concentration for extract was g %, for safranal volume (mL) of 0.2 mg mL^{-1} solution, and for theophylline mM. For saline only one volume (0.6 mL) was used. *$P < 0.05$, ***$P < 0.001$ compared with saline; †$P < 0.05$, ††$P < 0.001$ compared with theophylline; ‡‡$P < 0.01$ compared with safranal.

Table 3 Relaxant effects of aqueous-ethanolic extract from *Crocus sativus* in comparison with a negative control (saline) and a positive control (theophylline) in group 3 experiments (incubated preparations with 1 μM propranolol, 1 μM cholorpheniramine and 1 μM atropine contracted by 60 mM KCl, n = 6)

Different concn	Saline	Aqueous-ethanolic extract
0.15	–	1.84 ± 1.29
0.30	–	8.71 ± 3.60
0.45	–	17.96 ± 5.52
0.60	1.23 ± 0.62	26.48 ± 6.55*

Values are presented as mean ± s.e.m. The unit of concentration for extract was g %. For saline only one volume (0.6 mL) was used. *$P < 0.05$ compared with the effect of saline.

and theophylline (0.815 and 0.927 for groups 1 and 2) ($P < 0.001$ for all cases).

Discussion

In this study, the relaxant (bronchodilatory) effects of aqueous-ethanolic extract from *C. sativus* and safranal in comparison with saline (as negative control) and theophylline (as positive control) were studied. In group 1 (contracted tracheal chains by methacholine) all concentrations of theophylline extract and safranal showed potent relaxant effect on tracheal smooth muscle. In addition, extract from *C. sativus* and safranal showed relatively potent relaxant effects compared with the effect of saline in group 2 (contracted tracheal chains by KCl). In group 3, the extract of *C. sativus* showed a relatively weak relaxant effect compared with saline. The effects of theophylline and safranal were not examined in group 3 experiments.

The relaxant effect of extract, safranal and theophylline was concentration dependent. There were positive correlations between increasing concentrations and the relaxant effects of extract and safranal in all three groups of experiments. The relaxant effects of all concentrations of safranal were lower than those of the extract in groups 1 and 2. In addition, the effects of extract in group 1 and 2 experiments were similar to the effect of theophylline, but the effects of safranal were lower compared with theophylline.

The relaxant effect of extract from *C. sativus* and safranal on guinea-pig tracheal chains might have been produced due to several different mechanisms, including stimulation of β-adrenergic receptors, inhibition of histamine H_1-receptors or an anticholinergic property of the plant, because the relaxant effect of β_2-stimulatory (Linden et al 1993; Martin et al 1994), histamine H_1-receptor inhibitory (Popa et al 1984), and anticholinergic drugs (Loenders et al 1992) have been shown. To evaluate the contribution of β-adrenergic stimulatory, anticholinergic and/or H_1 histamine blocking effect of the aqueous-ethanolic extract from this plant on its bronchodilatory effects, the effects of these extracts on tracheal chains with inhibited β-adrenergic, muscarinic and histamine H_1 receptors by propranolol, atropine and chlorpheniramine,

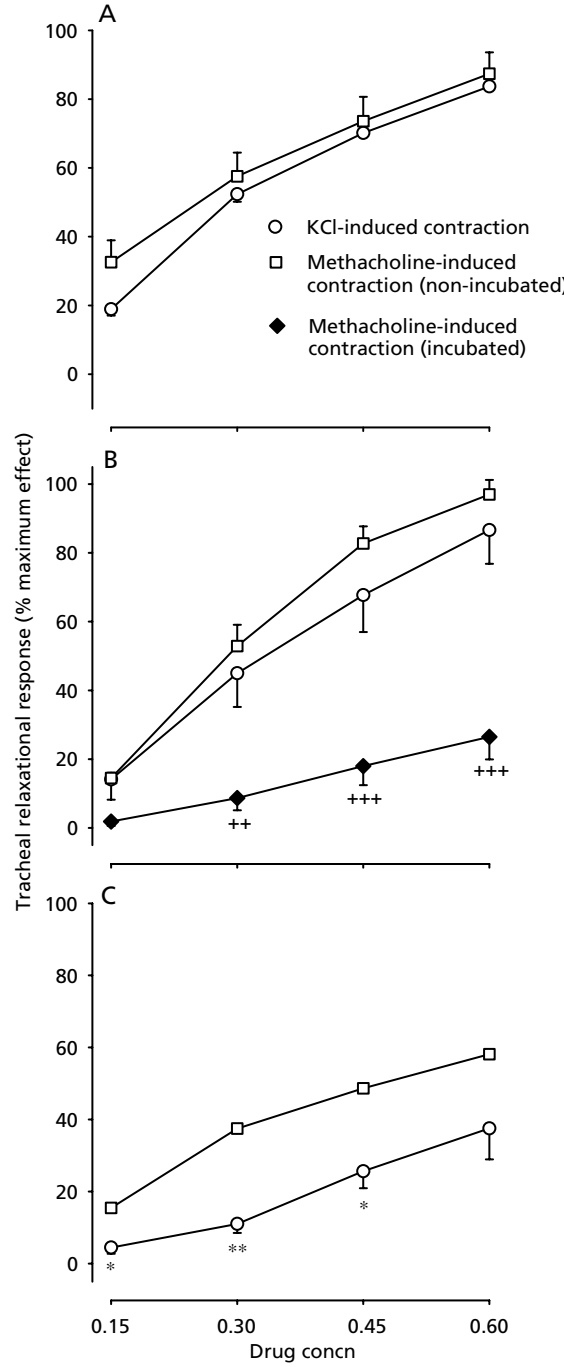

Figure 1 Concentration–response curves of the relaxant effect of (A) theophylline, (B) aqueous-ethanolic extract from *Crocus sativus*, and (C) safranal, in three groups of experiments. Group 1, methacholine-induced contraction on non-incubated tracheal chains (□); group 2, KCl-induced contraction on non-incubated tracheal chains (○); and group 3, KCl-induced contraction on guinea-pig tracheal chains incubated with propranolol, chlorpheniramine and atropine (◆) (n = 6 for each group). The unit of concentration for the extract was g %, for safranal volume (mL) of 0.2 mg mL^{-1} solution and for theophylline mM. *$P < 0.05$, **$P < 0.01$, statistical differences in the relaxant effect of different substances between groups 1 group 2. +$P < 0.05$, ++$P < 0.01$, +++$P < 0.001$, statistical differences in the relaxant effect of different substances between groups 1 and 2 with those of group 3.

respectively, were re-examined in group 3 experiments. The plant extract showed a weak relaxant effect in group 3 experiments. The relaxant effects of most concentrations of *C. sativus*-extract obtained in group 3 were significantly lower than those of group 2. These findings suggested probable β-adrenergic stimulatory, muscarinic and/or histamine H_1 blocking properties of the plant extract that may have contributed to the relaxant effect on guinea-pig tracheal chains. The enhanced relaxant effect of extract at higher concentrations, and positive correlation between its effect and concentration supported the competitive antagonist effect of the extract on muscarinic or histamine H_1 receptors and/or stimulatory effect on β-adrenoceptors. However, the most probable mechanism responsible for the relaxant effect of *C. sativus* on the tracheal chains was the stimulatory effect on β-adrenoceptors, because β-agonists to drugs are the most powerful bronchodilators.

While KCl affects calcium channels (Perez-Guerrero et al 1997) and with regard to the bronchodilatory effect of calcium-channel blockers (Miyahara et al 1993; McCaig & DeJonckeere 1993), the relaxant effect of *C. sativus* extract and safranal in group 2 (contracted tracheal chains by KCl) may have indicated a calcium-channel blocking effect of this plant and safranal. Another explanation for these findings was the absence of an opening effect of this plant and its main constituent safranal on potassium channels, because the bronchodilatory effect of potassium-channel opening drugs has been demonstrated previously (Buckle et al 1993). If the extract had a potassium-channel opening effect, it would not have relaxant effects on KCl-contracted tracheal chains, while it could show a relaxant effect on methacholine-contracted tracheal chains. In fact, the results of group 2 may support this effect of macerated extract.

The results of this study confirmed those of Fatehi et al (2003), indicating a relaxant effect of this plant on smooth muscles of the vascular system. However, in this study some mechanisms responsible for the relaxant effect of the plant, including β-adrenoceptor stimulatory, muscarinic or histamine H_1 inhibitory, calcium-channel inhibitory and potassium-channel opening effects, were explored.

The relaxant effect of safranal in groups 1 and 2 indicated that it was one of the main constituents of the plant responsible for the relaxant effect. However, the significant lower relaxant effect of safranal compared with extract on both groups of experiments suggested that other constituents of the plant might have contributed to the relaxant effect. Therefore, more studies are required to reveal the effective substances and extract mechanisms of action for the relaxant effect of *C. sativus*. The HPLC results showed that the extract contained only 0.026% safranal, while the amount of crocin in the extract was 41.3%. This suggests that the relaxant effect of crocin should be evaluated further.

With regard to the existence of airway inflammation in the tracheobronchial tree of asthmatic patients, *C. sativus* might have an anti-inflammatory effect, which would contribute to the therapeutic effect of this plant on asthma. In fact, the anti-inflammatory (Abe et al 1999) and antioxidant (Abdullaev 1993) effects of this plant have been shown. However, the effect of *C. sativus* on airway inflammation existing in asthma and other respiratory disease requires investigating.

Conclusion

The results indicated a relatively potent relaxant effect (bronchodilatory) of *C. sativus* on guinea-pig tracheal chains, which was comparable with theophylline. The results suggested that the relaxant effect of this plant could be due to β-adrenoceptor stimulatory, muscarinic and/or histamine (H_1) receptor inhibitory effect. A possible inhibitory effect of aqueous-ethanolic extract on calcium channels has been postulated. The results suggested that the safranal was, at least in part, responsible for the relaxant effect of the plant on tracheal chains.

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